DR. IRENE NEWTON (Orcid ID : 0000-0002-7118-0374)

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Title: Mitochondria and *Wolbachia* titers are positively correlated during maternal transmission

Running title: Correlation between mitochondria and *Wolbachia*

Authors: [1,2]Lucas P. Henry and [1]#Irene L.G. Newton

Affiliations: 1 = Department of Biology, Indiana University, Bloomington, IN;

2 = Currently at Department of Ecology and Evolutionary Biology, Princeton University, Princeton, NJ

#= corresponding, irnewton@indiana.edu

Abstract

Mothers provide their offspring with symbionts. Maternally transmitted, intracellular symbionts must disperse from mother to offspring with other cytoplasmic elements, like mitochondria. Here, we investigated how the intracellular symbiont *Wolbachia* interacts with mitochondria during maternal transmission. Mitochondria and *Wolbachia* may interact antagonistically and compete as each population tries to ensure its own evolutionary success. Alternatively, mitochondria and *Wolbachia* may cooperate as both benefit from ensuring the fitness of the mother. We characterized the relationship between mitochondria and *Wolbachia* titers in ovaries of *D. melanogaster*. We found that mitochondria and *Wolbachia* titers are positively correlated in common laboratory genotypes of *D. melanogaster*. We attempted to perturb this covariation through the introduction of *Wolbachia* variants that colonize at

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different titers. We also attempted to perturb the covariation through manipulating the female reproductive tract to disrupt maternal transmission. Finally, we also attempted to disrupt the covariation by knocking down gene expression for two loci involved in mitochondrial metabolism: NADH dehydrogenase and a mitochondrial transporter. Overall, we find that mitochondria and *Wolbachia* titers are commonly positively correlated, but this positive covariation is disrupted at high titers of *Wolbachia*. Our results suggest that mitochondria and *Wolbachia* have likely evolved mechanisms to stably coexist, but the competitive dynamics change at high *Wolbachia* titers. We provide future directions to better understand how their interaction influences the maintenance of the symbiosis.

Introduction

In order to ensure faithful transmission, vertically acquired symbionts must find their way to the next generation along with both nuclear and cytoplasmic genomes, like mitochondria. The cytoplasmic environment within a cell is long thought to be a competitive environment, creating conflict between the different resident genomes, particularly between maternally inherited, cytoplasmic genomes and the biparentally inherited, nuclear genome (Eberhard 1980, Cosmides and Tooby 1981, Greiner et al. 2015). Maternally transmitted, cytoplasmic genomes can evolve selfish strategies to circumvent competition within the cytoplasm and bias the transmission of their own genomes through increased replication at the expense of the host (Cosmides and Tooby 1981, Werren 2011). That said, given that both symbiont and mitochondrion rely on the evolutionary success of their hosts, cytoplasmic genomes sharing the same host should cooperate to facilitate their combined evolutionary success (Charlat et al. 2003, Frank 2003). Therefore, it is unclear if competition or conflict exists between mitochondria and intracellular symbionts during maternal transmission. Here, we leverage the experimentally tractable model host, *Drosophila melanogaster*, to investigate the interaction between mitochondria and an intracellular symbiont, *Wolbachia pipientis*.

Wolbachia are intracellular, α-proteobacteria that facultatively infect an estimated 40-60% of arthropods (Werren et al. 2008), including *D. melanogaster*. Both mitochondria and *Wolbachia* share the same, free-living ancestor in the α-proteobacteria clade (Wu et al. 2004). *Wolbachia* induce a variety of phenotypes in their hosts. *Wolbachia* are classically known as selfish genetic elements that manipulate host reproduction to maintain their infection in some host species (Werren et al. 2008, Werren 2011). However, *Wolbachia* also confer protection against viral pathogens in some host species (Hedges et al. 2008, Teixeira et al. 2008, Hoffmann et al. 2015). Many of the phenotypes induced by *Wolbachia* in *Drosophila* may depend on *Wolbachia* titer. *Wolbachia* titer is correlated with protection against virus pathogens (Osborne et al. 2012, Chrostek et al. 2013). However, high titers often result in reduced fitness for the host in the absence of a pathogen (Chrostek and Teixeira 2015, Martinez et al. 2015) and increased expression of cytoplasmic incompatibility leading to more effective reproductive manipulations by *Wolbachia* (Clancy and Hoffmann 1998, Veneti et al. 2004).

Mitochondria have evolved strategies to ensure their maternal transmission, often leading to competitive dynamics between different mitochondrial genotypes. For example, in *D. melanogaster*, mitochondria compete with other mitochondria over access to maternal transmission as shown in work with heteroplasmic flies. In heteroplasmic flies, mitochondrial genomes that gain a competitive advantage by increasing their replication at higher rates are transmitted from mother to offspring, regardless of fitness costs to the fly (Ma et al. 2014, Ma and O'Farrell 2016). This suggests that mitochondria respond to changes to its intraspecific, competitive environment, and furthermore, suggests that mitochondria might respond to challenges from other cytoplasmic elements. It is unclear how *Wolbachia* infection changes the mitochondrial dynamics during maternal transmission.

The evolutionary success of cytoplasmic genomes is ultimately determined by their ability to replicate and disperse from mother to offspring (Cosmides and Tooby 1981, Werren 2011). Therefore, both mitochondria and *Wolbachia* must occupy the eventual oocyte to ensure

maternal transmission. During maternal transmission, mitochondria and *Wolbachia* utilize similar mechanisms to ensure dispersal from mother to offspring. The cytoskeleton enables the establishment of polarity that is crucial for proper development of the oocyte (Verheyen and Cooley 1994b), and both mitochondria and *Wolbachia* use components of the cytoskeleton to ensure maternal transmission (Boldogh and Pon 2007, Serbus et al. 2008). Specifically, in flies, *Wolbachia* require host microtubules and both minus and plus-end motors (dynein and kinesin) for posterior localization in the mature oocyte, positioning themselves for inclusion in the germline of the next generation (Ferree et al. 2005, Serbus and Sullivan 2007). This suggests that access to the cytoskeleton is important to ensure maternal transmission, potentially creating competition between mitochondria and *Wolbachia*.

Few studies have characterized the interaction between mitochondria and *Wolbachia* during maternal transmission. First, coexistence and no competition is suggested by differing patterns of localization between mitochondria and *Wolbachia* during oogenesis in *D. melanogaster* observed through fluorescence microscopy (Ferree et al. 2005). Mitochondria localize to both poles, while only *Wolbachia* localize to anterior poles, suggesting they use different mechanisms to ensure maternal transmission. Second, in another study, mitochondria and *Wolbachia* genomic copy number was estimated from the number of reads that mapped to mitochondria or *Wolbachia* genomes from female *Drosophila simulans*, and mitochondria copy number was positively correlated with *Wolbachia* copy number (Signor 2017). The positive correlation measured in this study suggests a mutual beneficial coexistence such that hosts that are high quality habitats for mitochondria are also good for *Wolbachia.* However, as mentioned above, theory often predicts an antagonistic relationship between mitochondria and *Wolbachia*, which would suggest that the correlation of abundance between mitochondria and *Wolbachia* should be null or negative. Thus, quantifying the interaction between mitochondria and *Wolbachia* is important to understanding how these two cytoplasmic residents coexist during maternal transmission.

To understand potential interactions between mitochondria and *Wolbachia* titers during maternal transmission, we characterized titers of each cytoplasmic genome in fly ovaries. To do this, we performed four experiments: 1) We first characterized the relationship between mitochondria and *Wolbachia* titers in several common laboratory genotypes. Then, we cleared *Wolbachia* infection in flies to determine if *Wolbachia* limits mitochondria. 2) We manipulated the interaction between mitochondria and *Wolbachia* by using fly lines of the same host genotype but with high or low *Wolbachia* titers determined by *Wolbachia* genotype. 3) We attempted to manipulate the interaction by disrupting the development of the actin cytoskeleton in the ovaries. 4) Finally, we disrupted mitochondrial titers by knocking down two loci involved in mitochondrial metabolism and transport. Throughout our experimentation, we commonly found a positive covariation between mitochondria and *Wolbachia* titers in wild-type flies. The positive covariation between mitochondria and *Wolbachia* was disrupted for *Wolbachia* genotypes with high *Wolbachia* titers, but our other disruptions to the cytoskeleton or mitochondria did not alter the positive covariation.

Methods

Fly rearing and general experimental approach

We obtained all fly stocks from the Bloomington *Drosophila* Stock Center (BDSC) and Indiana University (see Supp. Table 1 for full genotypes). Standard methods were used for all crosses and culturing. Flies were reared on standard Bloomington food and at similar densities. All crosses were initiated within 5 days of eclosion so that parents were of a similar age across experiments. For all experiments, we sampled mitochondria and *Wolbachia* titers from ovaries dissected from mated females. Ovaries were obtained from female flies aged 3-5 days post eclosion. Before dissection, mated female flies were placed on grape juice agar plates for ~48 hrs with yeast paste to stimulate ovary development (Verheyen and Cooley 1994a, Wong and Schedl 2006). All flies were dissected on ice in cold, sterile DEPC-PBST under similar time

conditions. We determined mitochondria and *Wolbachia* titers only in the ovaries, as the female reproductive tissue is where maternal transmission occurs.

We primarily determined mitochondria and *Wolbachia* titers through quantitative PCR. We first extracted nucleic acids (DNA for all lines, both DNA and RNA for TRiP lines in Experiment 3) from ovaries from individual flies using a modified Trizol extraction protocol (see Supp. Methods for extended details). We acknowledge that measuring mitochondria titers may be complicated by the fission-fusion dynamics of mitochondrial replication. However, we attempted to control for this by only measuring mitochondria titer in one tissue, the ovary, dissected under controlled conditions. RNA and DNA were normalized after quantification to 15 ng per reaction for quantitative PCR.

Using quantitative PCR, we amplified three loci to investigate the ecology of maternal transmission. First, we determined changes in *Wolbachia* titer by amplifying *Wolbachia* surface protein, *wsp* (Forward: CATTGGTGTTGGTGTTGGTG; Reverse: ACCGAAATAACGAGCTCCAG). Second, we determined changes in mitochondrial titer by amplifying the mitochondrial genomic region adjacent to *COI,* by using *mtDNA* primers from (Zhu et al. 2014) (Forward: GATTAGCTACTTTACATGGAACTC; Reverse: CTGCTATAATAGCAAATACAGCTC). Third, to determine changes in the actin cytoskeleton, we amplified the *Drosophila* homolog to profilin, *Chickadee* (*chic*), by using *chic* primers from (Newton et al. 2015) (Forward: TGCACTGCATGAAGACAACA; Reverse: GTTTCTCTACCACGGAAGCG). We only measured *chic* expression in Experiment 3.

All quantitative PCR was performed independent of a host reference gene using standardized calibration curves. Calibration curves were generated using plasmids extracted from cloned amplicons to standardize for copy number based on plasmid + PCR insert (see Supp. Methods for more detail on procedure). This provided an absolute quantification of gene copy numbers present in each ovary sample based on C_T values. We performed qPCR reactions independent of host reference gene because the knockdowns created in Experiments 3 and 4

may also alter the host nuclear reference gene in unpredictable ways that might bias our measurement of mitochondria and *Wolbachia* titers. We used a single-copy host reference gene, *rpl32*, to measure mitochondria and *Wolbachia* titers in subset of ovaries; we found qualitatively similar patterns as the data collected independent of a host reference gene (see Supp. Methods).

All qPCR reactions were performed using an Applied Biosystems StepOne Real-time PCR system. For changes in mitochondria and *Wolbachia* titer, we amplified from DNA and used Fast SYBR-Green Master Mix (Applied Biosystems). For changes in *chic* expression, we performed qRT-PCR and amplified from RNA using SensiFast SYBR Hi-ROX One-step RT mix (BioLine). For the *w*Mel variant flies, we used the same cycling conditions, but with PowerUp SYBR Green Master Mix (Applied Biosystems). The plasmids were re-standardized for copy number to provide an absolute quantification of gene copy numbers for this master mix. We used this to determine mitochondria and *Wolbachia* titers only in the *w*Mel variant flies. Cycling conditions were as follows: 95ºC for 10 minutes, then 35 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. All samples were run in triplicate, and samples were rerun if C_T values for technical replicates had a standard deviation of greater than 0.5. C_T values generated were used to calculate absolute gene copy numbers, as described above, to determine mitochondria and *Wolbachia* titer. Gene copy numbers were then log2 transformed for normality.

Additionally, in Experiment 3, we validated our *Wolbachia* titer measurements using both Western blots and fluorescence microscopy (see Supp. Methods for details). These data show that our measurement of *Wolbachia* abundance is qualitatively similar across three different techniques. Below, we provide methods for four experiments to determine how mitochondria and *Wolbachia* interact during maternal transmission. We then describe the statistical analyses performed for each experiment. All statistical analyses were performed in R (R Development Core Team, 2008).

Experiment 1: Initial screening of common laboratory genotypes to determine mitochondria and *Wolbachia* association

We screened three common laboratory genotypes (w^1, *OreR*, w^{1118}) to determine if mitochondria and *Wolbachia* titers were correlated during maternal transmission. For these three genotypes, we generated *Wolbachia* positive and negative lines (i.e. w^1+/-). *Wolbachia* negative lines were generated by treating flies for two generations with 0.25 mg/mL tetracycline. After each tetracycline treatment, flies were reared with uninfected males of the same genotype to repopulate the microbiome. As tetracycline treatment itself might influence mitochondrial density (Ikeya et al. 2009), tetracycline-treated flies were reared for >5 generations on regular food, without antibiotics, before experiments. We dissected ovaries from age-matched females, extracted DNA, and performed qPCR to assay mitochondria and *Wolbachia* titers.

Statistical analyses: To determine if mitochondria titers differ between the three laboratory genotypes, we used a Kruskal-Wallis test. We compared the mitochondria titer between *Wolbachia* infected and uninfected lines within a genotype (i.e. w^1-*Wolbachia* positive versus w^1-*Wolbachia* negative) using Mann-Whitney U tests. We also compared *Wolbachia* titer between genotypes by Kruskal-Wallis test. To determine if mitochondria and *Wolbachia* titers are positively correlated, we regressed *Wolbachia* titer by mitochondria titer for each genotype. Additionally, to assess the influence of host genotype, *Wolbachia* infection, and the interaction of these factors on mitochondrial number we used a generalized linear model (GLM) to assess variation in mitochondria number between lines using fly genotype and Wolbachia presence as fixed effects, along with their interaction. The GLM was tested with both a Gaussian and a Gamma error distribution. The Gamma error distribution was a better fit for the data, which are not normally distributed. We used two additional statistical tests to assess variation in mitochondria load using fly genotype and *Wolbachia* presence as fixed effects, along with their

interaction: 1) a standard ANOVA, and 2) a permutational analysis of variance that assumes no error distribution (adonis, from the vegan package in R). We performed an ANCOVA to determine if slopes of covariation differed between genotypes.

Experiment 2: Examining covariation with different *Wolbachia* genotypes within the same host nuclear genotype

To alter the interaction *Wolbachia* and mitochondria in the same host genetic background, we utilized a panel of flies that have the same host background but differ only in their *Wolbachia* titer controlled by *Wolbachia* genotype (*w*Mel variant flies in Supplemental Table 1, from Chrostek et al. 2013). Briefly, to create these lines, Chrostek et al. isogenized chromosomes 1-3 in the DrosDel w^{1118} line using balancer chromosomes (Chrostek et al. 2013). Here, our goal was to control for nuclear background that might influence the environment within the ovaries. We reasoned that if *Wolbachia* and mitochondria compete, then high *Wolbachia* titers would limit mitochondria more than low *Wolbachia* titers. If so, we would observe differences in the slope of covariation.

However, because both mitochondria and *Wolbachia* are maternally transmitted, we cannot exclude that when the *Wolbachia* variants were selected, different mitochondria genomes were also selected. Chrostek *et al.* reported that mitochondria genotype did not influence the pathogen blocking phenotype induced by different *Wolbachia* genotype, but did not explore how genetic variation in the mitochondria segregated with different *Wolbachia* genotypes.

As in Experiment 1, we dissected ovaries from age-matched females, extracted DNA, and performed qPCR to assay mitochondria and *Wolbachia* titers.

Statistical analyses: We determined if different *Wolbachia* genotypes had different mitochondria titers using a Kruskal-Wallis test. We performed a similar analysis to determine if *Wolbachia* genotypes had different *Wolbachia* titers.

Because of small sample sizes, we grouped *Wolbachia* genotypes by phylogenetic cluster ,i.e. *w*MelCSa, *w*MelCS2a, *w*MelCS2b into *w*MelCS), to examine covariation between mitochondria and *Wolbachia* titers, which Chrostek et al. (2013) also do in their original analyses. First, because each *Wolbachia* genotype was introgressed into the same nuclear background, we could test if the nuclear genotype maintained the same positive covariation by regressing *Wolbachia* titer by mitochondria titer, irrespective of *Wolbachia* genotype. Second, we examined if the covariation differed between the three different groups of *Wolbachia* genotypes using ANCOVA.

Experiment 3: Manipulating mitochondria and *Wolbachia* titers by altering actin cytoskeleton

To determine a mechanism underlying *Wolbachia* and mitochondrial associations during maternal transmission, we performed crosses to modify the actin cytoskeleton in the female reproductive tissue. Previous work suggested that the actin cytoskeleton is used by both cytoplasmic entities (Ferree et al. 2005), and we reasoned that knocking down expression in specific tissues within the ovary may change the ecological dynamics between mitochondria and *Wolbachia*. To do this, we utilized fly stocks carrying UAS inducible short hairpin silencing triggers (TRiP) specific to profilin (BDSC #34523), derived from Ni et al. (2011). In order to knock down expression of the *Drosophila* profilin homolog *chickadee,* (abbreviated as *chic*), we used three different, tissue-specific drivers. The first tissue-specific driver, maternal triple

driver (MTD, BDSC #31777) is expressed uniformly and throughout oogenesis (Petrella et al. 2007). The second driver, *oskar* (OSK, BDSC #44241) is expressed during oogenesis under control of *osk* regulatory region involved in cell polarization (Kugler and Lasko 2009). The third driver, follicle cell driver (FCD, BDSC #7020) is expressed in the follicle cells at anterior and posterior poles of the egg chamber and in the embryonic epidermis (Attrill et al. 2015). We confirmed localization of the FCD to the poles of the egg chamber by crossing the FCD line to the a UAS:GFP line (Supp. Fig. 1).

To knockdown gene expression, *Wolbachia*-infected, homozygous TRiP females were crossed to homozygous males from each of the drivers. We also confirmed alterations to the cytoskeleton by staining with ActiStain-555 and visualizing at 60X using a Nikon E800 fluorescence microscope (see Supp. Methods for more detail).

We assayed *chic* transcripts, mitochondria and *Wolbachia* titers in ovaries from age-matched F1 females using qPCR. We also performed Western blots to confirm the qualitatively similar patterns in *Wolbachia* titer (see Supp. Methods for more detail). Finally, we performed fluorescence *in situ* hybridization (FISH) on TRiP parental females and TRiPxFCD knockdowns to further confirm patterns observed in *Wolbachia* titer (see Supp. Methods for more detail). We used the universal bacterial probe EUB388 conjugated to AlexaFluor 488 to detect *Wolbachia* localization during oogenesis as in Newton et al. (2015). While our EUB388 probe can bind to other bacteria species, the morphology and localization patterns were consistent with *Wolbachia* infection. We measured fluorescence intensity to quantify changes in *Wolbachia* localization. Briefly, images were taken as a Z-series stack at 1 um intervals at 60X oil objective using a Nikon NiE fluorescence microscope. Exposure times were controlled for all images taken per stage of oogenesis. Stacks were reconstructed in ImageJ. Metamorph was used to quantify fluorescence (see Supp. Methods for more detail).

Statistical analyses: For this experiment, we made individual comparison between the TRiP parental lines and each knockdown (i.e. TRiP parental versus TRiPxFCD knockdown). With the qPCR data, we compared *chic* transcript abundance, mitochondria titer, and *Wolbachia* titer between TRiP parental and each knockdown using Mann-Whitney U tests. We examined if the covariation in slope between mitochondria and *Wolbachia* titer differed using ANCOVA.

For the protein data obtained from Western blot, we determined if knockdowns had different *Wolbachia* titers compared to the TRiP parental line based on densitometric measures using a Mann-Whitney U test.

For the localization data obtained from microscopy, we compared pixel intensity scaled by area for the germaria, early oogenesis, and mid oogenesis between TRiP parentals and TRiPxFCD knockdowns using a Mann-Whitney U test.

Experiment 4: Manipulating mitochondria and *Wolbachia* by knocking down genes associated with only mitochondria function

Like in Experiment 3, we used fly lines carrying UAS inducible RNAi to alter the ecological interaction between mitochondria and *Wolbachia*. Here, we specifically knocked down genes associated with mitochondria function to reduce mitochondria titer and potentially increase *Wolbachia* titer, like competitive release. Specifically, we utilized fly stocks carrying UAS inducible short hairpin silencing triggers (TRiP) specific to NADH dehydrogenase (BDSC #36695) and a mitochondrial transporter (BDSC #34720). In order to knock down expression of the NADH dehydrogenase and mitochondrial transporter, we crossed these lines to the HSP70:GAL4 driver (BDSC #2077). The HSP70:GAL4 driver is known to be leaky in control of expression, affecting off-target gene expression (Newton et al., 2015)—so we did not perform a heat shock but instead compared flies with or without HSP70:GAL4 in the TRiP background.

Ovaries were dissected from age-matched F1 females, and we determined mitochondria and *Wolbachia* titers through qPCR.

Statistical analyses: We compared titers individually between the parental wild-type (without HSP70:GAL4 driver) to the mitochondria transporter and NADH dehydrogenase knockdowns separately. We used Mann-Whitney U test to determine if knockdowns differed by parental wild-type in mitochondria or *Wolbachia* titers. We performed ANCOVA to determine if the slope of covariation differed in response to each knockdown.

Results

Wolbachia and mitochondrial titers were correlated in laboratory genotypes

To investigate the interaction between cytoplasmic genomes during maternal transmission, we first surveyed *Wolbachia* and mitochondrial titers in the ovaries of three common laboratory genotypes, w^1, *OreR*, and w^{1118}, using qPCR. We cleared each line of *Wolbachia* infection, creating six lines. We found that genotypes differed in mitochondrial titer (Supp. Fig. 2, Kruskal-Wallis χ^2 =12.502, df=5, p=0.002), but lines did not differ significantly within a genotype when infected with *Wolbachia* (Fig 1A, w^1+/w^1-: Mann-Whitney U=41, p=0.9654; *OreR*+/-: Mann-Whitney U=18, p=1; or *w1118*+/-: Mann-Whitney U=20, p=0.4286). We assessed variation in mitochondria number between lines using fly genotype and *Wolbachia* presence as fixed effects, along with their interaction. We used a GLM (with both a Gaussian and a Gamma error distribution), a standard ANOVA, and a permutational analysis of variance that assumes no error distribution. For all models, there was no significant effect of the interaction between *Wolbachia* presence and fly nuclear genotype on mitochondrial load and no significant effect of *Wolbachia* presence on mitochondrial load (Supp. Table 2). However, for all models we

found a significant effect of genotype alone on mitochondrial load. Therefore, our analysis suggests that host genotype alone influences mitochondrial titers.

Wolbachia titers differed between the three genotypes, with *OreR* flies exhibiting highest titers (Fig. 1B, Kruskal-Wallis χ^2=11.61, df=2, p=0.003); titers for *OreR* were ~2-fold higher than w^1 and ~6-fold higher than w^{1118}. Mitochondria and *Wolbachia* titers tended to be positively correlated in both w^1 (β=0.24, p=0.003, r^2=0.736) and *OreR* (β=0.1, p=0.059, r^2=0.63), but not in *w1118* (β= -0.2668, p=0.722, r^2=0.17). However, the slopes of covariation did not differ among genotypes (Fig. 1C, $F_{2,13}$=1.883, p=0.19), suggesting that the overall trends is for mitochondria and *Wolbachia* titers to be positively correlated.

Wolbachia genotypic difference alters positive covariation with mitochondria

Because *Wolbachia* and mitochondria occupy the cytoplasm and may have evolved strategies to bias intracellular resources to ensure their own transmission, we speculated that mitochondria and *Wolbachia* might interact over maternal transmission. If they interact, we reasoned they might limit each other to ensure their own spread. To manipulate this interaction, we utilized *w*Mel variant flies of the same host genetic background, but differing only in their *Wolbachia* genotypes, which vary in titer (Chrostek et al. 2013). We reasoned if *Wolbachia* and mitochondria compete over maternal transmission, then high titer *Wolbachia* genotypes would have a different slope of covariation with mitochondria than low titer *Wolbachia* genotypes.

First, we determined that different *Wolbachia* genotypes infect the host at different titers (Fig. 2A, Kruskal-Wallis χ^2=16.31, df=6, p=0.01); the *w*Au and *w*MelCS variants establish high titer infections, while *w*Mel variants establish lower titer infections, as previously described in Chrostek et al. (2013) (Fig. 2A). Interestingly, the same pattern was observed for

mitochondrial titers (Fig. 2B, Kruskal-Wallis χ^2=17.03, df=6 p=0.009). Because each *Wolbachia* genotype infected the same maternal nuclear genotype, we could determine that the host genotype maintains positive covariation between mitochondria and *Wolbachia* titers (individual *Wolbachia* genotypes visualized in Supp. Fig. 3, β=0.85, p<0.001, r^2=0.70). While we did not have the statistical power to evaluate the differences in slope for each *Wolbachia* genotype, we found no significant difference between *Wolbachia* strains of the same clade; *w*Mel genotypes (*w*Mel2a, *w*Mel2b, *w*Mel3) do not differ in mitochondria titer (Kruskall-Wallis χ^2= 3.2967, df=2, p=0.19) or *Wolbachia* titer (Kruskall-Wallis χ^2= 4.4967, df=2, p=0.11) and *w*MelCS genotypes (*w*MelCS2a, *w*MelCS2b, *w*MelCSa) do not differ in mitochondria titer (Kruskall-Wallis χ^2= 1.5316, df=2, p=0.46) or *Wolbachia* titer (Kruskall-Wallis χ^2= 1.0038, df=2, p=0.61). Therefore, we grouped together *Wolbachia* genotypes by phylogenetic association, as in Chrostek et al. (2013); each of these variants are nearly genetically identical, with only a handful of SNPs separating them (Richardson et al., 2012). *w*MelCS flies had different slope of covariation than *w*Au or *w*Mel (Fig. 2C, $F_{2,33}$= 5.310, p=0.01). *w*MelCS flies had higher *Wolbachia* titers, and the slope of covariation between mitochondria and *Wolbachia* was not significantly different from zero (β =0.05, p=0.81, r^2=0.003). The loss of covariation between mitochondria and *Wolbachia* in *w*MelCS suggests that high titer *Wolbachia* genotypes can alter interactions with mitochondria during maternal transmission.

Attempting to intensify competition through disrupting the actin cytoskeleton in female reproductive tract does not disrupt mitochondria-*Wolbachia* positive covariation

To better characterize the interaction underlying the positive covariation between mitochondria and *Wolbachia* during maternal transmission, we manipulated host genetic factors using short-hairpin RNAi previously identified as potentially limiting for *Wolbachia*.

First, we knocked down the expression of one actin regulatory protein, profilin, in three different tissues within the female reproductive tract. We confirmed that the knockdown of profilin resulted in different gene expression of the profilin homolog, *chickadee*, in different

tissues the ovaries (Supp. Fig. 4A, Kruskal-Wallis χ^2=13.84, df=3 p=0.003). However, only TRiPxMTD had significantly reduced gene expression (TRiPxMTD: Mann-Whitney U=28, p=0.006). We then examined the ovaries using fluorescent microscopy to determine qualitative changes to actin cytoskeleton resulting from the knockdown. TRiP parental and TRiPxFCD lines exhibited normal, wild-type actin cytoskeleton, while TRiPxMTD and TRiPxOSK knockdowns had severe cytoskeleton deformities (Supp. Fig. 4B-E). By knocking down the expression of profilin in these tissues, we altered the actin cytoskeleton.

We reasoned that if both mitochondria and Wolbachia utilized the actin cytoskeleton, then disrupting the actin cytoskeleton would change their titers in the female reproductive tract. We did not detect any changes to *Wolbachia* titers in response to profilin knockdown compared to the TRiP parental line through Western blotting (Fig. 3A, Supp. Table 3 for statistics) or through qPCR (Fig. 3B, Supp. Table 3 for statistics). Mitochondrial titers were not affected in profilin knockdowns compared to TRiP parental lines either (Fig 3C, Supp. Table 3 for statistics). If profilin were a limiting resource that might influence the covariation between mitochondria and *Wolbachia* titers, then both mitochondria and *Wolbachia* titers would correlate with profilin transcripts. *Wolbachia* titer was not correlated with profilin transcripts (Fig 3D, Supp. Table 4 for statistics), nor was mitochondria titer (Fig. 3E, Supp. Table 4 for statistics). This suggests that *Wolbachia* and mitochondria titers did not respond to our manipulation of the actin cytoskeleton in the reproductive tract. These results suggest other mechanisms determine the association between *Wolbachia* and mitochondria in the female reproductive tract.

We hypothesized that if *Wolbachia* and mitochondria compete over the actin cytoskeleton to disperse, we would create stronger competition by limiting profilin through the knockdowns. If either *Wolbachia* or mitochondria were more strongly limited than the other by profilin, then the positive covariation in titers would be disrupted. However, we find that *Wolbachia* titers were still positively correlated with mitochondrial titers (Fig. 3F, Supp. Table 4

for statistics). Interestingly, the slope of positive covariation only significantly differed between TRiP and TRiPxOSK knockdowns ($F_{1,8}$=6.464, p=0.034).

Because we observed the trend for slight increases in *Wolbachia* titer when we reduced profilin expression within ovaries, we utilized fluorescence microscopy to confirm patterns observed by qPCR and Western blots. First, we examined if the disruption of the actin cytoskeleton altered *Wolbachia* localization during oogenesis, comparing the TRiP parental line and the TRiPxFCD knockdown. We focused on the TRiPxFCD knockdown because it did not suffer the severe defects of morphology observed when we used the other drivers (Supp. Fig 4) and therefore we would not conflate the aberrant oogenesis defects with *Wolbachia* localization anomalies. We used FISH targeting the 16S rRNA gene of *Wolbachia* and examined the ovaries by confocal microscopy to find qualitative differences in localization (Supp. Fig 5). In the TRiP parental line, *Wolbachia* are distributed homogenously in the germaria and at mid-oogenesis (Supp. Fig. 5A-C). In contrast, in the TRiPxFCD knockdown, beginning in the germaria, *Wolbachia* are clustered in micro colonies within the host tissue and not homogenously distributed through the ovariole in early oogenesis (Supp. Fig. 5D-F). We also examined *Wolbachia* localization in a small number of both TRiPxMTD (Supp. Fig. 6) and TRiPxOSK (Supp. Fig. 7) knockdowns, and found qualitatively similar clustering in micro colonies. These qualitative observations suggest that even the subtle knockdown of profilin results in alterations to *Wolbachia* localization at the beginning of oogenesis.

We then quantified changes in localization using fluorescence microscopy comparing the TRiPxFCD knockdown to the TRiP parental line. We found increased *Wolbachia*, based on fluorescence intensity, within the germarium of the TRiPxFCD progeny (Fig. 4A, Mann-Whitney U=213, p<0.001); fluorescence intensity was 1.5-fold higher in TRiPxFCD germaria. The increase in *Wolbachia* we observed was maintained through early oogenesis (approximately stage 3, Fig. 4B, Mann-Whitney U=281, p=0.039). However, during mid-oogenesis, at approximately stage 6, *Wolbachia* levels do not differ between TRiPxFCD knockdown and the

TRiP parental line (Fig. 4C, Mann-Whitney U=264, p=0.258). These findings suggest that *Wolbachia* responds to altered actin cytoskeleton through changes in localization during maternal transmission, but, combined with qPCR data, likely does not change the ecological interaction with mitochondria.

Knockdown of mitochondrial titers did not affect *Wolbachia* titers

To create an environment to potentially release *Wolbachia* from competition with mitochondria, we knocked down two genes that influence mitochondrial metabolism—a mitochondrial transporter and NADH dehydrogenase. We hypothesized that if mitochondria and *Wolbachia* compete during oogenesis, reducing mitochondria titer should result in an increase in *Wolbachia* titer, like competitive release. Mitochondria titers were significantly reduced in the two knockdowns compared to the parental wild type (Fig. 5A). Mitochondria titers in the mitochondria transporter knockdown were reduced by 86% (Mann-Whitney U=0, p<0.001), and by 71% for the NADH dehydrogenase knockdown (Mann-Whitney U=1, p<0.001). However, *Wolbachia* titers were not significantly affected (Fig. 5B). *Wolbachia* titers in the mitochondria transporter knockdown tended to increase (Mann-Whitney U=62, p=0.055) and were not significantly different in the NADH dehydrogenase knockdown (Mann-Whitney U=57, p=0.15). The knockdowns did not alter the positive slope of covariation between mitochondria and *Wolbachia* titers (Fig. 5C). Because mitochondria titers were reduced, the intercepts changed, but slope did not for either mitochondria transporter knockdown ($F_{1,14}$=1.540, p=0.23) or for the NADH dehydrogenase knockdown ($F_{1,14}$=0.239, p=0.632).

Discussion

Intracellular symbionts and mitochondria have similar evolutionary interests: to disperse from mother to offspring. We sought to determine how mitochondria and the intracellular symbiont, *Wolbachia*, interact during maternal transmission. We found that mitochondria and *Wolbachia* titers commonly positively covary across different laboratory *Drosophila* genotypes and different *Wolbachia* genotypes in the ovaries (Fig. 1D, Fig. 2C, Fig. 3F,

Fig. 5C). We disrupted the positive covariation in genotypes of *Wolbachia* that have high titer (Fig. 2C). When we perturbed the actin cytoskeleton, we only altered the slope of covariation in one knockdown, TRiPxOSK, but slopes were always positive (Fig. 3C). When we reduced mitochondria titer through RNAi, we saw a trend for slight increases in *Wolbachia* titers, but no change in the slope of covaration (Fig. 4C). Our results suggest that generally mitochondria and *Wolbachia* titers are positively correlated, but the positive covariation can be disrupted when *Wolbachia* titers are high.

To our knowledge, no other study has demonstrated this positive covariation between mitochondria and *Wolbachia* titer in the ovaries of *D. melanogaster* or other arthropods. We believe that we accurately measured the covariation between mitochondria and *Wolbachia* titers. We quantified genome copy number of mitochondria and *Wolbachia* for titer in the ovaries using qPCR, and found qualitatively similar patterns through Western blots (Fig. 3A), and microscopy for the TRiP and TRiPxFCD lines (Fig. 4). Additionally, we do not believe that this positive covariation is an artifact of differences in DNA quantity across samples because we normalized by DNA quantity after RNAse treatment. Additionally, we quantified mitochondria and *Wolbachia* titers relative to a host reference gene, *rpl32*, in a subset of ovaries from the TRiP knockdown experiment. We still observed the positive correlation between mitochondria and *Wolbachia* titers in the ovaries (Supp. Fig. 8).

Our goal was to characterize the nature of the interaction between mitochondria and *Wolbachia* during maternal transmission. If they compete with each other, we expected several outcomes. First, if *Wolbachia* were limiting mitochondria populations, we would see higher mitochondria titers in ovaries from uninfected flies. We did not detect any statistically significant differences in mitochondria titer between infected and uninfected ovaries (Fig. 1A-B). Other studies also found no effect of clearing *Wolbachia* infection on mitochondria titer in *D. melanogaster* ovaries (Touret et al. 2014) or in whole *Leptopilina* parasitoid wasps (Mouton et al. 2009). Second, we reasoned that if we reduce mitochondria populations that limit *Wolbachia*,

we would expect to see an increase in *Wolbachia* populations. We did not detect any significant increase in *Wolbachia* titer when we knocked down genes associated with mitochondria despite a reduction in mitochondria titer (Fig. 5). While there was a trend for increases in *Wolbachia* titer for the mitochondria transporter knockdown, the effect size was small. Interestingly, we observed differences across fly genotypes with regards to *Wolbachia* and mitochondrial titer. *Wolbachia* and mitochondrial titers were significantly different between fly genotypes, with titers varying by 2 and 6 fold for *Wolbachia* (Fig. 1). Although we did not identify the host genes responsible for the difference in titer, a recent study in the *Nasonia* system suggests a single locus may regulate symbiont titers, named *Wolbachia* density suppressor (*Wds*) (Funkhouser-Jones et al., 2018). No homolog for *Wds* is found in the *Drosophila* genome but it is possible that a single locus, or a few loci, may also explain differences in we observe across the genotypes investigated here. Future work should identify whether the same locus that regulates *Wolbachia* also influences mitochondrial titers.

We used loci previously identified in a genome-wide RNAi screen of *Drosophila* cell lines infected with *Wolbachia* (White et al. 2017). White et al. also performed validations in *Drosophila* using the same two knockdowns we used—however, in their study, only *Wolbachia* titers were measured, not mitochondria. Importantly, White et al. observed different effects—when NADH dehydrogenase was knocked down, *Wolbachia* titers increased. When mitochondrial transporter was knocked down, *Wolbachia* titers decreased. However, we observed no significant reductions in *Wolbachia* titers for either gene (Fig. 5). White et al. quantified *Wolbachia* titers using propidium iodide staining and microscopy, rather than with quantitative PCR like we did here, which may explain the differences in the response of *Wolbachia* titer to mitochondria knockdowns in our study.

Both of the genes knocked down in mitochondria are associated with mitochondrial metabolism. Mitochondria generate energy that is used by other organelles in the cell; we hypothesized that *Wolbachia* would rely on energy generated from mitochondria, and changes

in the transport of ATP from mitochondrion to the cytoplasm would change *Wolbachia* titers, as suggested by White et al. (2017). Because we did not detect changes in *Wolbachia* titer in response to the likely reduction in ATP transport to the cytoplasm, *Wolbachia* may require little energy from the mitochondria or manipulates the host in some way to ensure its own persistence. *Wolbachia* may be more limited by other kinds of resources, as other studies have identified changes in *Wolbachia* titer in response to macronutrients derived from exposure to different diets (Caragata et al. 2014, Ponton et al. 2015, Serbus et al. 2015). However, while differential resource limitation may explain niche partitioning here, the environmental niche that may differentiate *Wolbachia* and mitochondria remains unknown.

Our experiments to manipulate the actin cytoskeleton were designed to test if mitochondria and *Wolbachia* demonstrated niche partitioning over access to the actin cytoskeleton. If mitochondria and *Wolbachia* require access to the actin cytoskeleton to ensure localization to the oocyte, then reducing the amount of actin in certain tissues would result in decreases in mitochondria and *Wolbachia* titers because both rely on the actin cytoskeleton to reach the developing oocyte (Boldogh and Pon 2007, Serbus et al. 2008, Newton et al. 2015). Previously, we showed that when profilin is present in a hemizygous condition, *Wolbachia* suffers significant defects in colonization of the ovaries and subsequently, reductions in transmission (Newton et al. 2015, Newton and Sheehan 2015). Our manipulations did not succeed in significantly changed titers of either mitochondria or *Wolbachia* (Fig. 3B-C).

However, when we knocked down *oskar* in the TRiPxOSK line, we changed the slope of covariation between mitochondria and *Wolbachia* titers. We reasoned that if the ecological interactions change, then we would see changes in the slope of covariation between mitochondria and *Wolbachia* titers. Indeed, for the TRiPxOSK knockdown, the slope is steeper than the TRiP parental lines, i.e. *Wolbachia* titer increases more per mitochondria increase. *oskar* is implicated in maintaining polarity during oogenesis (Kugler and Lasko 2009). *oskar* interacts with components of the cytoskeleton that leads to polar localization of the

mitochondria necessary for maternal transmission (Cox and Spradling 2003). *oskar* also interacts with a complex suite of mRNAs to ensure polarization, such as *gurken* (Kugler and Lasko 2009). Manipulating *gurken* altered polarization patterns, leading to increases in *Wolbachia* titer (Serbus et al. 2011). From our data, the steeper slope of covariation may suggest that the TRiPxOSK knockdown has increased the proliferation of *Wolbachia* relative to mitochondria. While we did not examine in detail localization in the TRiPxOSK knockdown or interactions with *gurken* or other genes involved in ensuring polar localization, future work would be necessary to understand how altered localization between mitochondria and *Wolbachia* may alter their ecological interaction.

Interestingly, we found that genotypes of *Wolbachia* with high *Wolbachia* titers disrupted the positive covariation with mitochondria, suggesting that their ecological interaction is altered (Fig. 2C). We reasoned that when positive covariation occurs, this suggests that both mitochondria and *Wolbachia* are both benefiting from inhabiting the environment, such that larger populations of both cytoplasmic entities are supported. However, for the high titer *w*MelCS strains, the positive covariation no longer exists, suggesting that large populations of *Wolbachia* likely increase resource limitation within the cytoplasm, changing the intracellular environment, altering the interaction between mitochondria and *Wolbachia*.

Our findings of positive covariation between mitochondria and *Wolbachia* titers, except at high titers of *Wolbachia*, suggest some interdependence where *Wolbachia* modify the ovarian environment in a way that also benefits mitochondria indirectly. However, at high *Wolbachia* titers, the environment is no longer modified to benefit both. *Wolbachia* commonly alters the host cellular environment in the female reproductive tract of *Drosophila* (Fast et al. 2011, Serbus et al. 2011, Christensen et al. 2016, Sheehan et al. 2016, Rice et al. 2017). These alterations should ensure that *Wolbachia* can replicate and proliferate, but should also ensure that the female reproductive tract successfully produces female offspring. Mitochondria may also benefit from these modifications. As mentioned previously, in a genome-wide RNAi screen

of *Drosophila* cell lines infected with *Wolbachia*, knockdown of several other genes involved in mitochondrial function also resulted in changes to *Wolbachia* titers (White et al. 2017). In White et al., the responsiveness of *Wolbachia* to changes in the gene expression of mitochondria could suggest interdependence, though White et al. did not measure mitochondria response. Mitochondria respond to different microenvironments within the female reproductive tract, and these different microenvironments can favor the replication of different mitochondrial genomes in *D. melanogaster* (Ma et al. 2014). Future work should investigate if *Wolbachia* modifications of the female reproductive tract may also provide benefits to mitochondria replication and transmission.

Finally, mechanisms that maintain ecological coexistence may explain the positive covariation between mitochondria and *Wolbachia* titers. First, mitochondria and *Wolbachia* may not interact directly during maternal transmission, as their joint goal is successful maternal transmission (Charlat et al. 2003). To facilitate their joint evolutionary success, mitochondria and *Wolbachia* may coexist, not interacting directly with each other. To stably coexist, both must limit their own populations more than they limit each other (Chase and Leibold 2003). Coexistence suggests both mitochondria and *Wolbachia* have evolved strategies to minimize competition and direct interactions between each other, limiting their interaction and partitioning the niche of maternal transmission. Our data support the scenario of limited interaction, as we show no difference in mitochondria titer between infected and uninfected *Wolbachia* lines (Fig. 1A-B). Coexistence is also suggested from mitochondria knockdowns. *Wolbachia* titers did not change in response to decreased mitochondria titers (Fig. 5A-B), and this is suggestive of niche partitioning over metabolic products in the cytoplasm. However, high titer *Wolbachia* genotypes have altered the cytoplasmic environment that might disrupt stable coexistence. *w*MelCS genotypes disrupt the positive slope of covariation, likely shifting to more competitive and antagonistic interactions with mitochondria.

Ultimately, though, the host nuclear genotype ensures a positive covariation between mitochondria and *Wolbachia* (Fig. 2C, Supp. Fig. 3). The maintenance of positive covariation is likely in the evolutionary interest of the nuclear genome to ensure stable coexistence between the cytoplasmic residents. Interestingly, high titer *Wolbachia* strains often reduce host longevity in the absence of pathogen pressure, suggesting evolutionary instability (Chrostek and Teixeira 2015, Martinez et al. 2015). While we did not examine longevity in this study, the interaction between mitochondria and *Wolbachia* may explain some of the fitness costs. Indeed, in the original description of high titer strain *w*MelPop, which phylogenetically clusters with *w*MelCS strains (Chrostek et al. 2013), mitochondria were observed to deteriorate as *Wolbachia* densities increased through electron microscopy (Min and Benzer 1997). From an evolutionary perspective, if high *Wolbachia* titers result in shortened lifespan, then strong selection may exist for the nuclear genome to maintain *Wolbachia* titers that ensure positive covariation with mitochondria titers. Future studies investigated the natural standing variation in wild flies or other arthropods infected with *Wolbachia* would provide critical insights to the evolutionary pressures that shape mitochondria-*Wolbachia* interactions.

In conclusion, we find mitochondria and *Wolbachia* titers are positively correlated during maternal transmission in *D. melanogaster*. This may suggest coexistence through limiting their interaction and potential mutual benefits to each other. However, the interaction changes in the context of high *Wolbachia* titers, disrupting the positive covariation and potentially intensifying competition. The biology of mitochondria and *Wolbachia* is likely intricately intertwined within the cell, and their interactions remain underexplored (Correa and Ballard 2016). Furthermore, many other intracellular symbionts induce phenotypes like *Wolbachia* (Engelstädter and Hurst 2009), and these interactions within the cytoplasm likely influence the evolutionary trajectory of the host (Rand et al. 2004). The interaction between mitochondria and intracellular symbionts may provide better understanding of how the evolution of cooperation or selfishness influences the maintenance of maternally transmitted symbioses.

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